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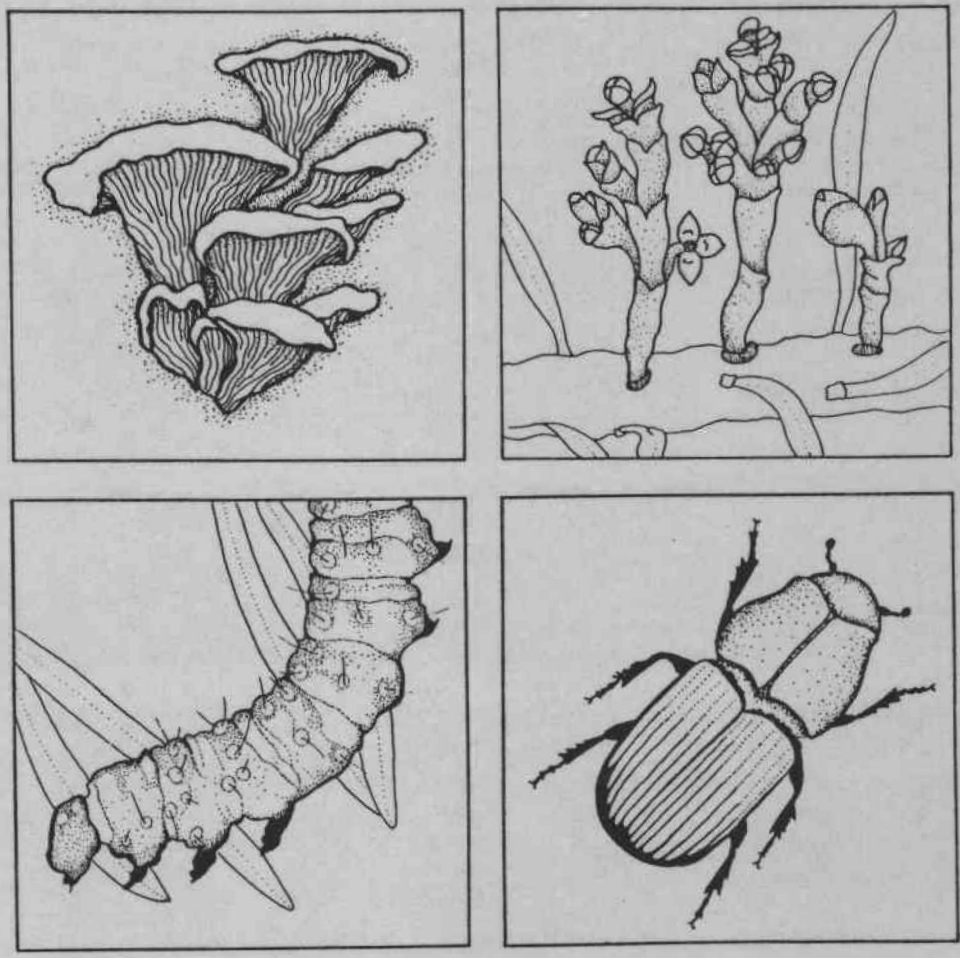

PATHOGENICITY OF *FUSARIUM* FROM FOREST SEEDLING NURSERIES ON DOUGLAS-FIR AND PONDEROSA PINE SEEDLINGS

by

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ABSTRACT

Eighteen isolates of Fusarium comprising four species (F. oxysporum, F. avenaceum, F. acuminatum, and F. sambucinum) isolated from diseased conifer seedlings from nurseries were tested for pathogenicity on Douglas-fir germlings. Three of the most pathogenic F. oxysporum isolates on Douglas-fir germlings were also tested on ponderosa pine germlings and older Douglas-fir seedlings; three of the most pathogenic F. sambucinum isolates on Douglas-fir germlings were likewise tested on older Douglas-fir seedlings. Most F. oxysporum isolates and all the other fusaria were highly pathogenic to germlings. Level of virulence on germlings was best measured by rate of infection and tissue degradation. All inoculated older Douglas-fir seedlings became infected, although foliar disease symptoms were rare. Based on extent of root system colonization, isolates of F. oxysporum were generally more pathogenic to older Douglas-fir seedlings than F. sambucinum. These tests confirm the wide range of pathogenicity of fusaria commonly isolated from diseased seedlings.

INTRODUCTION

Fusarium spp. are frequently isolated from the roots of conifer seedlings in nurseries. They may be associated with a wide range of disease symptoms (James 1985b) or with healthy appearing seedlings (Bloomberg 1966; James and Gilligan 1984). Therefore, occurrence of Fusarium on seedlings does not necessarily mean that it is pathogenic. Factors mediating pathogenic activity of these fungi involve both host responses and environmental effects.

To more clearly define the role of Fusarium as a conifer seedling pathogen, standard tests evaluating pathogenicity are needed. This report summarizes two tests used to quantify pathogenicty and elucidate levels of virulence of several Fusarium isolates obtained from conifer seedlings in nurseries.

MATERIALS AND METHODS

Isolates of Fusarium tested in this study were obtained mostly from diseased conifer seedlings from several northern Rocky Mountain forest tree nurseries (Table 1). Eighteen isolates were evaluated on germlings in aseptic test tube inoculations and six of the most pathogenic were further tested on older and larger potted seedlings.

Test Tube Inoculations--Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) and ponderosa pine (Pinus ponderosa Laws.) seed were surface sterilized in 40 percent bleach solution (2.1 percent aqueous sodium hypochlorite) for 10 minutes and washed thoroughly under running tap water for 4 days in order to remove contaminating fungi. Seed were then stratified at 0 to 3°C for 7 days. After stratification, seeds were sown in plastic petri dishes lined with four layers of sterile, moist filter paper and incubated at about 21°C under an 8-hour light and 16-hour darkness regime. Germlings were used for inoculations when they were 10 to 15 mm in length (about 3 to 5 days old).

Table 1.--Description of Fusarium isolates tested for pathogenicity on Douglas-fir and ponderosa pine seedlings.

Isolate designation	Species[1]	Host		Nursery
		Species[2]	Tissue	
82-59F	FOXY	DF	Seed	USDA For. Serv., Coeur d'Alene, ID
83-42A	FOXY	DF	Roots	Montana State, Missoula, MT
83-59A	FOXY	DF	Seed	Montana State, Missoula, MT
83-71-10	FOXY	WF	Roots	Fantasy Farms, Peck, ID
83-73	FOXY	DF	Roots	USDA For. Serv., Coeur d'Alene, ID
83-74	FOXY	DF	Roots	USDA For. Serv., Coeur d'Alene, ID
84-11	FOXY	DF	Roots	Montana State, Missoula, MT
84-21	FOXY	ES	Roots	Alpine, Kalispell, MT
84-22-D2	FOXY	-	Soil Mix[3]	Alpine, Kalispell, MT
82-56G	FSAM	RO	Stems	Montana State, Missoula, MT
82-57B	FAVE	DF	Seed	USDA For. Serv., Coeur d'Alene, ID
73-1F	FACU	PP	Needles	Montana State, Missoula, MT
83-51A	FSAM	PP	Roots	Dan Day, Kalispell, MT
83-67 #1	FSAM	LP	Roots	Alpine, Kalispell, MT
83-91A #1	FSAM	PP	Stems	USDA For. Serv., Albuquerque, NM
84-13-B3	FACU	-	Soil Mix[3]	Montana State, Missoula, MT
84-21 #1	FACU	ES	Roots	Alpine, Kalispell, MT
84-22 D3	FAVE	-	Soil Mix[3]	Alpine, Kalispell, MT

[1]FOXY = Fusarium oxysporum; FAVE = Fusarium avenaceum;
FACU - Fusarium acuminatum; FSAM = Fusarium sambucinum

[2]DF = Douglas-fir; WF = white fir; ES = Engelmann spruce; RO = Russian-olive; PP = ponderosa pine; LP = lodgepole pine.

[3]Standard peat-vermiculite soil mix used to grow containerized seedlings; samples were from mixes within which diseased conifer seedlings were growing.

Test tubes for inoculations were prepared by placing two layers of folded filter paper within each tube (to provide a platform away from the bottom of tubes to support germlings and inoculum), followed by 6 ml sterilized, distilled water; covering with a foam plug; and sterilizing by autoclaving. Within each sterilized test tube, a plug of inoculum about 5 mm in diameter, (1-week-old culture grown on potato dextrose agar (PDA)) was aseptically placed on the folded filter paper. Control treatments consisted of test tubes with agar plugs only. Germlings were aseptically placed in test tubes so that their roots were in contact with the inoculum. Each isolate was replicated 10 times. Inoculated and uninoculated (control) germlings were incubated at 21°C under a diurnal regime of 8 hours of light and 16 hours of darkness.

Germlings were examined periodically for disease symptoms. The experiment was terminated after 22 days at which time isolations were made from the roots of germlings. Isolates obtained were compared with the original inoculum, which was maintained on PDA slants, to determine if they were the same.

All isolates were tested on Douglas-fir germlings. But because of poor ponderosa pine seed germination, only three of the most pathogenic F. oxysporum Schlect. isolates on Douglas-fir germlings (82-59F, 83-74, 84-21) were tested on pine germlings.

Douglas-fir Pot Inoculations--Six of the more pathogenic isolates from the test tube inoculations (82-56G; 82-59F; 83-42A; 83-51A; 83-67 #1; 83-73) were tested on containerized Douglas-fir seedlings by inoculating the potting medium. These seedlings were 8 months old and had been grown in styroblock containers.

Inoculum consisted of a peat-perlite-Czapek agar mixture. Inoculum was prepared by mixing 200 ml Czapek agar solution with 1,500 mg peat-perlite mixture and autoclaving for 30 minutes. When cooled, 1,200 ml of this agar mixture was combined with the contents of three culture tubes of the test fungus (7-day-old cultures) which had been macerated in 300 ml sterile water. After inoculation, the mixture was incubated for 2 weeks at 24°C. Following incubation, 200 ml of the inoculum mixture was combined with 1,200 ml of a sterilized peat-vermiculite growth medium placed in pots, into which test seedlings were transplanted. Controls consisted of adding noninoculated Czapek-peat-perlite mixtures to sterilized peat vermiculite. Care was taken to wash seedling root systems thoroughly and avoid wounding during transplanting. Twelve seedlings were inoculated with each test fungus. Inoculated seedlings were maintained on greenhouse benches and checked periodically for symptoms.

Eleven months after inoculation, all seedlings were analyzed for infection. A five-class rating system was formulated for estimating severity of foliar symptoms on inoculated seedlings (Table 2). Procedures for quantitatively estimating extent of root system colonization by Fusarium are summarized in Table 3. These procedures allowed a fairly accurate estimate of the degree of virulence as measured by level of root system colonization. Isolates recovered from roots were compared with the original inoculum to determine if they were the same.

Table 2.--Foliar symptom classifications of Douglas-fir seedlings inoculated with Fusarium.

Class	Symptoms[1]
1	Greater than 80% of foliage green and healthy
2	50-80% of foliage green
3	30-50% of foliage green
4	10-20% of foliage green
5	Less than 10% of foliage green

[1] Nongreen foliage was chlorotic or necrotic.

Table 3.--Procedures for estimating amount of infection of Douglas-fir seedlings with Fusarium.

1.	Wash entire root system under tap water to remove loose soil adhering to roots.
2.	Surface sterilize root system in a bath of 4.0 percent bleach (0.21 percent aqueous sodium hypochlorite) for 4 minutes.
3.	Rinse sterilized root system thoroughly under tap water to remove excess bleach.
4.	Randomly select 10 lateral roots and aseptically cut them from the main tap root.
5.	Cut tips (about 0.5 cm in length) from the 10 selected roots and aseptically place on selective medium (Komada 1975).
6.	Cut a piece from the top of selected roots (where they join the main tap root) and aseptically place on selective medium.
7.	Incubate plates for 7 days at about 24°C under a 12-hour diurnal light-darkness regime.
8.	Count the number of root pieces infected with Fusarium and calculate: (a) percent of roots, (b) percent of root tips and (c) percent of root junctions infected.

Histological Studies--Infected germlings from the test tube inoculation experiment were sectioned where the main tap root came into contact with inoculum. Four of the isolates considered most pathogenic (82-59F, 83-42A, 83-74, and 84-21) because they killed all germlings within 22 days of inoculation, were evaluated. Tissues were fixed in formalin-acetic acid alcohol (FAA), dehydrated in tertiary butyl alcohol, and embedded in paraffin. After embedding, tissues were cut with a rotary microtome at 12 u and stained with Feulgens and fast green. Sections were examined under the light microscope (100-450X) for hyphal interactions with host cells.

RESULTS

Test Tube Inoculations--Six of the nine isolates of F. oxysporum were considered highly pathogenic (Table 4), i.e., they killed all Douglas-fir germlings within 22 days of inoculation. Most of these isolates initially penetrated roots and began tissue degradation within 2 to 3 days. Less pathogenic isolates killed only 40 to 70 percent of the germlings and usually took longer (12 to 15 days) to infect and degrade host tissues than highly pathogenic isolates. The three F. oxysporum isolates tested against ponderosa pine germlings (82-59F, 83-74, 84-21) were also pathogenic to this species. Inoculated pine germlings were invaded quickly and killed within 22 days. Control germlings were not killed.

Table 4.--Susceptibility of Douglas-fir germlings to selected isolates of Fusarium oxysporum.

Isolate[1]	Percent germlings killed after 22 days[2]
82-59F	100
83-42A	100
83-73	100
83-74	100
84-21	100
84-22D3	100
83-59A	40
83-71-10	60
84-11	70
Controls	0

[1] See Table 1 for isolate description.

[2] Most pathogenic isolates (100 percent of germlings killed) caused initial infection and degradation of roots within 2 to 3 days. The less pathogenic isolates took longer (12 to 15 days) for infection and degradation to occur.

All the other Fusarium species (F. avenaceum, F. acuminatum, F. sambucinum) were highly pathogenic, invading and killing Douglas-fir germlings within 4 to 5 days of inoculation.

Douglas-fir Pot Inoculation--Results of inoculating containerized Douglas-fir seedlings with six of the most pathogenic isolates tested on germlings are summarized in Table 5. All seedlings became infected with the inoculated isolates. In general, F. oxysporum isolates colonized a greater portion of the seedling root system than the F. sambucinum isolates. Low average symptom ratings indicated that many infected seedlings lacked noticeable foliar symptoms. Relationships between amount of root system infection and intensity of foliar symptoms were not apparent in this study. Several noninoculated control seedlings became infected with Fusarium, although amount of root infection was low. Seedlings selected for inoculation probably had a low level of "background" infection. Since the fungi reisolated from inoculated seedlings generally corresponded to those inoculated, apparently these "background" fusaria were readily replaced by the inoculated isolates.

Histological Studies--Microscopic examinations of sections of primary roots where they came into contact with inoculum in germling test tube inoculations showed that invasion of roots by F. oxysporum was very rapid. Twenty-four hours after inoculation the fungus had entered the roots and in some samples had spread to cells on the outer edges of the vascular or central root cylinders (figure 1). Three days after inoculation, most isolates had spread to all root cells and some cortex cells were beginning to deteriorate (figure 2). Six days after inoculation all root cells were completely invaded by F. oxysporum and usually extensively deteriorated (figure 3).

Table 5.--Pathogenicity of selected Fusarium isolates to containerized Douglas-fir seedlings.

Isolate	Species	Average symptom rating[1]	Percent infection			
			Seedlings	Root tips	Root joints	Root system[2]
82-59F	F. oxysporum	1.9	100.0	90.8	77.5	84.2A
83-42A	F. oxysporum	1.7	100.0	95.0	77.5	86.3A
83-73	F. oxysporum	1.8	100.0	99.2	91.7	95.4A
82-56G	F. sambucinum	1.4	100.0	75.8	61.7	68.8B
83-51A	F. sambucinum	2.0	100.0	63.3	40.8	52.1B
83-67 #1	F. sambucinum	1.2	100.0	75.8	59.2	57.5B
Control	--	1.2	58.3	19.2	8.3	13.8C

[1] See Table 2 for description.

[2] Estimate of the overall percentage of the root system infected (average of root tips and root joints). Values followed by the same capital letter are not significantly different (P = 0.05) using Duncan's Multiple Comparison Test.

Figure 1.--Taproot cross-section of a Douglas-fir germling invaded by Fusarium oxysporum (isolate 82-59F) 24 hours after inoculation (X250). Root epidermis is at the top and the central vascular cylinder is at the bottom. Fungal hyphae had readily colonized cortical cells between the epidermis and central vascular cylinder.

Only a limited number of pictures were available

Figure 2.--Taproot cross-section of a Douglas-fir germling invaded by Fusarium oxysporum (isolate 83-74) 72 hours after inoculation (X250). Root epidermis is to the left and the central vascular cylinder is to the right. Fungal hyphae had penetrated to the vascular cylinder and a few cortical cells had deteriorated.

Figure 3.--Taproot cross-section of a Douglas-fir germling invaded by Fusarium oxysporum (isolate 84-21) 144 hours after inoculation (X250). Root epidermis is at the top and the central vascular cylinder is at the bottom. All root cells are completely colonized by fungal hyphae and extensive root deterioration is evident.

DISCUSSION

Our studies confirmed pathogenic variability of individual Fusarium species from conifer seedlings (Armstrong et al. 1940; Gordon 1965; James and Gilligan 1984; Wellman and Blaisdell 1941). Pathogenic variability may be related to differential host responses (i.e., host resistance) and inherent genetics of fungal pathogenicity (Gordon 1965). Several workers (Armstrong et al. 1940; Harvey 1929; Wellman and Blaisdell 1941) have attempted to correlate level of pathogenicity with cultural characteristics such as growth rate and tendency to produce abundant aerial mycelium. Our experience thus far indicates that virulent isolates cannot consistently be predicted on the basis of stable cultural characteristics. Inoculation tests on conifer hosts are apparently required.

Inoculations of young conifer germlings in sterile test tubes have previously been successful in differentiating pathogenic Fusarium isolates (James 1985c; Tint 1945). Advantages of this system include rapidity of test results and the limited resources needed to conduct such tests. However, our results on Douglas-fir and ponderosa pine indicated that only the least pathogenic isolates were differentiated in germling inoculation tests. Although pathogenic and saprophytic isolates can usually be separated in germling tests, the level of virulence may be obscured because of large inoculum potential. Therefore, rate of infection and tissue degradation are probably better indicators of level of virulence than assessment of mortality rates. We would expect the more virulent isolates to infect and colonize germlings more rapidly than less virulent isolates. On the basis of rapidity of infection and tissue degradation, we were able to identify the most virulent F. oxysporum isolates. However, germling tests were unable to differentiate isolates of the other Fusarium species tested (F. avenaceum, F. acuminatum, F. sambucinum). All of these species rapidly invaded and killed germlings.

To more closely simulate disease conditions expected in a nursery, inoculation tests on larger seedlings were necessary. Although such tests take longer to assess, they often provide a better basis for separating virulent and nonvirulent fusaria. Ideally, inoculations would consistently separate pathogenic from saprophytic isolates on the basis of level of seedling mortality and symptom expression. However, in our tests most infected seedlings lacked foliar disease symptoms even though roots of all inoculated seedlings became colonized with test isolates. Previous work (Bloomberg 1966) indicated that Fusarium often invades conifer seedling roots without eliciting host responses such as foliar symptoms. Other studies (James 1984a; James 1984b) also indicated that seedlings may be infected for extended periods without displaying symptoms. However, during periods of seedling stress, such as limiting nutrients and water during bud set in container operations, fusaria may become aggressive and cause seedling decline with associated symptoms (James 1985b). In our studies, inoculated seedlings were maintained for 11 months because they generally lacked disease symptoms. However, we suspect that seedling roots probably became infected shortly after inoculation. Abundance of nutrients and moisture added to test seedlings may have reduced symptom expression, or the isolates tested may not have been aggressive enough to consistently overcome host defenses. Therefore, we based our assessment of virulence mostly on extent of root system colonization. The more aggressive isolates would probably colonize a greater portion of the root system. Isolates classified as virulent on the basis of root system colonization did

not necessarily cause the most severe disease symptoms; i.e., level of root colonization was not related to amount of foliar symptom production. On the basis of root system colonization, the F. oxysporum isolates tested were more pathogenic than the F. sambucinum isolates.

Some outplanted seedlings infected with Fusarium may be considered "healthy" because of lack of disease symptoms. Smith (1967) showed that F. oxysporum on seedling roots is readily replaced by other soil fungi when infected seedlings are outplanted on forest soils. However, other work (James 1985a) indicates that if much of the root system is infected, stress induced by storage or outplanting may be sufficient to cause extensive mortality of planted seedlings during the first growing season.

Our results indicated that most of the F. oxysporum isolates tested were pathogenic to conifer seedlings. This confirms other work (Gordon 1965; James and Gilligan 1984; Matuo and Chiba 1966), although levels of virulence may vary widely. The other tested fusaria were originally classified as F. "roseum" based on older taxonomic schemes of Snyder and Hansen (Toussoun and Nelson 1968). This grouping was unsatisfactory because many of the organisms classified as the same species behaved quite differently. Therefore, more modern and acceptable taxonomic schemes (Booth 1971; Gerlach and Nirenberg 1982) were used to separate isolates into three species: F. avenaceum, F. acuminatum, and F. sambucinum. In the past, many F. "roseum" isolates have been considered saprophytic on conifer seedlings (Buxton et al. 1962; Vaartaja and Cram 1956). However, other work (James and Gilligan 1984; Matuo and Chiba 1966; Morgan 1983) indicates that F. "roseum" may be very pathogenic to conifer seedlings at times. In our tests, the F. "roseum" isolates were very pathogenic on germlings, but less so on older Douglas-fir seedlings.

Another problem arises because of common changes in Fusarium isolates due to mutations. Such changes include colony morphology and probably affect pathogenicity. Therefore, level of pathogenicity may change over time depending on the amount of isolate mutation that occurs.

In conclusion, our work indicated that the fusaria commonly associated with conifer seedling diseases includes organisms that vary in their ability to incite diseases. Further work is needed to elucidate roles of separate species when several fusaria are isolated from individual diseased plants. Organism succession or synergistic relationships among various fungi may be involved. Continued research should receive high priority because of the widespread impact of Fusarium-associated diseases in conifer nurseries and the relatively high value of affected hosts.

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